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Characterization of a Stable L-Form of *Bacillus subtilis* 168

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A stable L-form of *Bacillus subtilis* 168 (*sal-1*) has been isolated which grows and divides logarithmically in liquid medium with a generation time of 60 min. This mutant does not synthesize cell wall as evidenced by chemical, biochemical, and morphological analyses. Antibiotics which specifically inhibit cell wall biosynthesis do not affect the growth of the L-form. Significant differences exist between the membrane proteins of the bacillary form and the L-form. The relative profile of membrane proteins varies with the salt concentration of the medium in both the L-form and the bacillary form.

The discovery of L-forms of *Streptobacillus moniliformis* in 1935 by Klieneberger (19) provided a novel system for the study of the role of the cell wall in the maintenance of the bacterial cell. By investigating the reversion of L-forms it is possible also to explore the requirements for recovery of surface polymers. There have been numerous studies concentrating on the structure of the L-form, the mode of cellular division, the relationship between L-forms and mycoplasma, and the chemical composition of the cytoplasmic membrane (for reviews, see 9, 13, 14, 30). The development of techniques by Landman and co-workers for the production of L-forms and the reversion of L-forms in *Bacillus subtilis* led to a model system in which the biochemical genetics of the L-form state could be analyzed (7, 21, 22, 26, 35). Until recently, these elegant studies were hampered by the lack of stable L-forms which could grow and divide in liquid medium. Now the combination of mutagenesis, removal of the cell wall with lysozyme, propagation of the surviving clones on agar, and adaptation of these clones to growth in liquid medium has resulted in the isolation of mutants which grow and divide in liquid medium with a generation time of approximately 60 min (45). The present study demonstrates that one of these mutants, *sal-1*, is devoid of cell wall constituents, is resistant to many of the antibiotics which inhibit the biosynthesis of the cell wall, and displays differences in the proteins of the cytoplasmic membrane when compared to the bacillary form. This paper was presented at the 72nd Annual Meeting of the American Society for Microbiology, Philadelphia, Pa., 23-28 April 1972.

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MATERIALS AND METHODS

Strains. The parent strain, *B. subtilis* BR151 (carrying *lys-3*, *trpC2*, and *metB10*), and the L-form mutant *sal-1* were used in this study (45).

Media. The L-form, *sal-1*, was grown in T-medium (in grams per liter: tryptone [Difco], 3.0; glucose, 1.0; KH_2PO_4, 1.0; K_2HOP_4, 3.0; NaCl, 70; and sufficient 1 N NaOH to adjust the *p*H to 6.8-7.0) at 37 C in a New Brunswick G-25 gyratory shaker at 250 rev/min. Occasionally the NaCl concentration was reduced and the survivors were subcultured at lower osmotic pressure. The SDS plating medium originally described by Landman and Forman for viable counts of L-forms (21) was modified for this study. The L-form medium (LF medium) contains in grams per liter: NH_4NO_3, 1.0; $(NH_4)_2SO_4$, 0.1; KH_2PO_4, 1.5; K_2HPO_4, 3.5; glucose, 2.0; L-tryptophan, 0.05; D-methionine, 0.05; agar (Difco), 8.0; sodium succinate, 90.0; and $MgCl_2$, 0.5. This medium was supplemented with 20 ml of heat-inactivated (56 C, 30 min) horse serum (Microbiological Associates). In all experiments the carbon source was autoclaved separately.

Bacillary cultures were grown in Spizizen minimal medium supplemented with glucose, casein hydrolysate, and the auxotrophic requirements as described previously (45), or in antibiotic medium no. 3 (Penassay, Difco).

Analysis of growth of L-forms. *sal-1* was grown overnight in a New Brunswick G-25 shaker at 250 rev/min in a 50-ml flask containing 4 ml of T-medium at 37 C. The next day, 0.1 ml of an overnight culture was added to 10 ml of T-medium, and 0.1 ml of this diluted culture was used to inoculate flasks contain-

ing 4 ml of T-medium. The flasks were placed in a 37 C incubator and shaken at 250 rev/min. At the time of inoculation and at 2-hr intervals thereafter, 0.1 ml of culture was removed from each flask and diluted in T-medium, and four 0.01-ml samples from each appropriate dilution were spotted on LF medium. The petri dishes were incubated for 3 days at 37 C in a moist chamber. The colonies within 4 drops from each dilution were counted under a dissecting microscope at 15× magnification to determine the number of colony-forming units (CFU)/ml of culture. In some experiments, vancomycin (obtained from Eli Lilly and Co.), novobiocin (purchased from Merck and Co.), bacitracin, chloramphenicol, D-cycloserine, or penicillin G (obtained from Sigma Chemical Co.) was added as indicated.

Microscopy. The procedures used for phase microscopy (12) and electron microscopy (45) were similar to those previously used in this laboratory.

Fractionation of L-forms to detect cell wall polymers. The L-form was grown at 37 C to a cell density of 10^8 CFU/ml in 1 liter of T-medium containing 1.2 M NaCl at 250 rev/min. The culture was harvested by centrifugation at 10,000 × *g* for 15 min at 4 C. The supernatant fluid was lyophilized, and the pellet was fractionated by the Park and Hancock procedure (32). The cell pellet (140 mg dry weight) was suspended in 40 ml of 5% trichloroacetic acid and incubated for 8 min in a 90 C water bath. The suspension was sedimented at 5,000 × *g* for 10 min and the supernatant fluid was discarded. The cells were resuspended in 40 ml of 75% ethanol, incubated for 20 min at 25 C, and subjected to centrifugation as before. The pellet was resuspended in 40 ml of buffer containing 0.05 M NH_4HCO_3, 0.005 M NH_4OH, and trypsin (0.1 mg/ml, Worthington Biochemical Co.) and was incubated for 2.5 hr at 37 C. After centrifugation at 5,000 × *g* for 10 min, the pellet (2.7 mg dry weight) was washed twice with distilled water, concentrated by lyophilization, resuspended in 2 ml of 6 N HCl and hydrolyzed at 102 C for 11 hr.

The lyophilized supernatant fluid was resuspended in 100 ml of cold 5% trichloroacetic acid and allowed to stand for 30 min at 4 C. Particulate material was sedimented at 5,000 × *g* for 15 min, and the supernatant fluid was extracted four times with ether to remove the trichloroacetic acid and then concentrated by lyophilization. The concentrate was resuspended in 140 ml of distilled water, and a 20-ml sample was placed on a column (45 by 2.5 cm) of Sephadex G-25 (Pharmacia Fine Chemicals, Inc.) and eluted with distilled water at 5 C. Fractions of 9 ml were collected. The absorbency of the fractions was determined at 240 nm and those showing an absorbency were assayed for the presence of *N*-acetylhexosamines (34).

Accumulation of nucleotide-bound murein precursors in sal-1. Four 1-liter cultures of logarithmic cells of *sal-1* were harvested at 10,000 × *g* for 10 min. The cell pellets (100 to 120 mg dry weight) were resuspended in 125 ml of T-medium without antibiotic or with penicillin G (1,000 μg/ml), vancomycin (50 μg/ml), or bacitracin (5 μg/ml). The suspensions of *sal-1* were incubated at 250 rev/min for 90 min at 37 C, sedimented at 10,000 × *g* for 10 min, and resuspended in 25 ml of cold 5% trichloroacetic acid. The cytoplasm of the suspensions of *sal-1* was extracted and analyzed for bound *N*-acetylamino sugars by the procedure of Chatterjee et al. (4). A logarithmic culture of the bacillus in 500 ml of Penassay medium (300 mg dry weight) was harvested by centrifugation, resuspended in 125 ml of Penassay medium containing 20 μg of vancomycin/ml, incubated, and extracted by the same procedure used for the L-form.

Preparation of membranes. Cultures of the bacillary form grown under the conditions given in the text were harvested by centrifugation at 8,000 × *g* for 5 min at 4 C, and the cells were resuspended in 40 ml of 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (*p*H 8.0) containing 10 mM $MgCl_2$ and 1.0 mM ethylenediaminetetraacetic acid. Lysozyme (10 mg) and 0.4 mg each of ribonuclease B and deoxyribonuclease (Worthington Biochemical Co.) were added to the mixture which was then incubated for 20 min at 37 C. Lysozyme was omitted when membranes were prepared from L-form cultures. Digestion of cell walls of the bacillary form was monitored by phase microscopy and spectrophotometrically (585 nm, 1-cm path length) by the decrease in turbidity due to lysis upon dilution in distilled water. After digestion for 15 min, the suspension of membranes and wall fragments was centrifuged at 4,000 × *g* for 5 min at 4 C to pellet membrane aggregates and intact cells. The membranes in the supernatant fluid were sedimented by centrifugation at 22,000 × *g* for 15 min at 4C. The pellet was resuspended in 35 ml of buffer and sedimented at 22,000 × *g*. This step was repeated twice. The membranes were finally resuspended in 4 to 10 ml of buffer, and 0.5-ml samples were distributed into small vials and stored at −20 C. Each membrane preparation was thawed and used only once.

SDS-polyacrylamide gel electrophoresis. The procedure of Weber and Osborn (41) was used. Gels (90 by 5 mm) containing 7.5% acrylamide were polymerized in 100 by 5 mm (inner diameter) glass tubes. Membrane preparations were thawed and adjusted to a protein concentration of 1.7 mg/ml, and 100 μliters of each membrane suspension was placed in a 6 by 50 mm tube. Dissociation mixture (50 μliters) containing equal proportions of 10% sodium dodecyl sulfate (SDS), 10% 2-mercaptoethanol, and sodium phosphate buffer (0.8% NaH_2PO_4, 3.85% $Na_2HPO_4 \cdot H_2O$, 0.2% SDS) was added to each tube. The membrane solutions were placed in a boiling-water bath for 1 min to dissociate the membrane protein (1) and cooled to room temperature, and 20 μliters of a marker mixture (45% sucrose [w/v] and 0.03% bromophenol blue) was added. This resulted in preparations with final membrane protein concentrations of 1 mg/ml. After 20 to 80 μg of each solubilized membrane protein was layered over the top of a gel, electrophoresis was performed at 6 ma/tube at 20 C for 3 to 4 hr. The gels were removed from the tubes and stained overnight with a filtered mixture containing: 10% trichloroacetic acid, 10% methanol (v/v), 0.13% Coomassie brilliant blue (Mann Research Labs., New York), and 0.25% aniline blue black

(Allied Chemical, Morristown, N.J.). The gels were destained in 10% trichloroacetic acid and scanned at 600 nm in a Gilford linear transport coupled to a Gilford model 2400-S spectrophotometer. The intensity of staining was recorded on a 0 to 1.0 absorbency scale.

Analytical procedures. Protein was estimated by the procedure of Lowry et al. (24) with bovine serum albumin as standard. Membrane protein determinations involved adding one drop of 10% SDS to ensure solubilization of membrane proteins. The *N*-acetylamino sugars were assayed by the method of Reissig et al. (34). Amino acid and amino sugar analysis was performed on a Technicon TSM analyzer. Results were computed from standards as described previously (12). All ^{14}C radioactivity counting determinations were made with a Beckman model LS-230 liquid scintillation counter by using the aqueous scintillation fluid containing Triton X-100 (Emulsion Engineering Inc., Elk Grove Village, Ill.), described by Patterson and Greene (33).

RESULTS

Growth of sal-1 in liquid medium. The L-form was initially grown at 37 C on a rotary shaker at 60 rev/min in flasks containing T-medium (46). Subsequently, *sal-1* was adapted to growth at 250 rev/min by inoculating a flask containing 10 ml of T-medium with 0.5 ml of *sal-1* grown at 60 rev/min. The flask was incubated at 37 C at 250 rev/min until visible turbidity was observed (7 days). Routinely, the L-form was transferred every 3 days into 4 ml of T-medium by using a loopful (0.01 ml) of culture for inoculum. Growth at 250 rev/min reduced the amount of cell clumping which was occasionally noticed at slower speeds. T-medium usually contained 1.2 M NaCl to provide osmotic stabilization for the L-form. This requirement could be reduced to 0.34 M NaCl by subculturing *sal-1* in medium containing successively lower concentrations of NaCl.

Growth was followed by determining the number of CFU with the dilution-drop procedure described in Materials and Methods. Reproducible results were obtained because the L-form colonies appeared as raised white dots, 1 mm in diameter with no "fried egg" morphology when viewed through a dissecting microscope against a dark background. The upper curve in Fig. 1 is typical of the growth of *sal-1* at 37 C in T-medium containing 1.2 M NaCl. After a 2-hr lag period, logarithmic growth continues for 8 hr with a generation time of 60 min to a cell density of 10^8 CFU/ml. Direct cell counts in a Petroff-Hausser bacteria counter also gave reproducible results. The number of L-form cells per milliliter calculated by this method was twofold greater than the viable count values. Attempts to follow growth by changes in culture turbidity were not practical. When an absorbency of 0.05 (585 nm, 1-cm light path) was detected, there were 3×10^7 CFU/ml of culture. One absorbency unit corresponded to 5.5×10^8 CFU/ml or 0.25 mg/ml (dry weight) Therefore, by employing direct cell counts or viable counts, we were able to follow L-form growth with better reproducibility than has been obtained in the past with other L-forms.

Cell wall polymers in cultures of sal-1. Because the synthesis of peptidoglycan is not obligatory in stable L-forms (18, 31), a major objective was establishing whether *sal-1* could synthesize cell wall polymers. Constituents of the cell wall (diaminopimelic acid [Dpm] and muramic acid) were not detected in either the

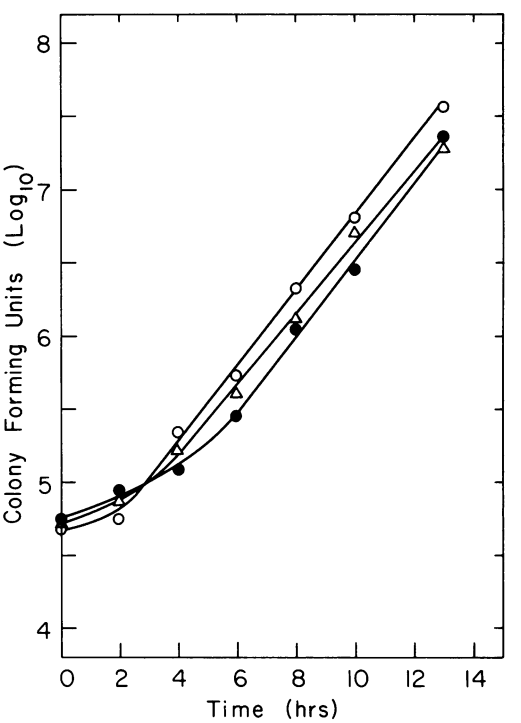

FIG. 1. *Effect of antibiotics on the growth of sal-1 in liquid medium. An overnight culture was diluted 200-fold, and 0.1 ml was used to inoculate 50-ml flasks containing 4 ml of T-medium containing 1.2 M NaCl without antibiotic (O) or with 1,000 μg of penicillin G per ml (●) or 50 μg of vancomycin per ml (Δ). The flasks were incubated at 250 rev/min at 37 C. Samples were removed at the time of inoculation (zero hr) and at 2-hr intervals for viable counts by the procedure outlined in Materials and Methods. The points on the curves represent combined results from duplicate experiments.*

supernatant fractions or cell pellets from 1-liter cultures of *sal-1*. The analysis of the cell pellets (described in Materials and Methods) would have detected as little as 10 nmoles of muramic acid per 100 mg dry weight.

In another experiment, the bacillary and L-forms were grown at 250 rev/min for 20 hr at 37 C in 10 ml of Penassay medium or T-medium, respectively, containing α, ϵ-diaminopimelic acid-*1*,7-^{14}C (0.1 μCi/ml, specific activity of 0.3 mCi/mmole, International Chemical and Nuclear Corp.) and 100 μg of L-lysine per ml. An excess of unlabeled L-lysine was added to the medium to prevent the incorporation of label into protein by decarboxylation of Dpm to lysine. Cultures of the bacillus (2×10^9 cells/ml) and L-form (3×10^8 cells/ml) were harvested by centrifugation at 5,500 $\times$ *g* for 10 min at 4 C, resuspended in 3 ml of 5% trichloroacetic acid, and extracted by the Park and Hancock procedure (32). At the start of fractionation, unlabeled cells from an overnight culture of the bacillus were added to provide a cell pellet of workable size. Incorporation of label into the various cell fractions of the bacillus and L-form is presented in Table 1. The amount of ^{14}C-Dpm in the trypsin-insoluble fraction of *sal-1* is negligible by comparison with the bacillus, suggesting that the L-form does not incorporate Dpm into a cell wall polymer. More label was incorporated into the ethanol-soluble fraction of the L-form than the bacillus.

Effect of antibiotics on the growth of sal-1. The results of chemical analysis indicated that *sal-1* did not synthesize cell wall polymer. Therefore, antibiotics that block cell wall synthesis would not be expected to inhibit the growth of the L-form. This supposition was tested by determining the minimal growth-inhibitory concentrations (MIC) of various antibiotics for the bacillus and L-form. Tubes containing serial dilutions of each antibiotic in 5 ml of Penassay medium for the bacillus or T-medium for *sal-1* were incubated at 37 C for 48 hr at 60 rev/min on a roller drum. The results are presented in Table 2. The concentrations of D-cycloserine, penicillin G, and vancomycin required to inhibit the growth of the L-form were many times greater than for the bacillus, but bacitracin and novobiocin were more inhibitory for *sal-1*. The bacillus and L-form were equally sensitive to chloramphenicol, an inhibitor of protein synthesis.

The growth rate of *sal-1* was determined by viable counts in T-medium containing antibiotic. Chloramphenicol, at a concentration of 1 μg/ml, did not allow growth of *sal-1*, but the viability remained constant for 13 hr (data not shown). Neither penicillin G nor vancomycin at the concentrations used caused a reduction in the generation time of *sal-1* (Fig. 1). D-Cycloserine did not produce a decrease in the growth rate of *sal-1* until the MIC level (500 μg/ml) was approached (Fig. 2). Bacitracin caused an immediate drop in viability at a concentration of 4 μg/ml (Fig. 3). After a 6-hr lag period, L-form growth resumed at the normal rate. Novobiocin, at a concentration of 0.2 μg/ml, caused an immediate cessation of growth (Fig. 4), but viability remained constant for a few hours before lysis. When novobiocin (final concentration of 0.2 μg/ml) was added to a logarithmically growing culture, growth stopped immediately (Fig. 4). D-Cycloserine and penicillin G,

TABLE 1. *Incorporation of ^{14}C-diaminopimelic acid into cellular fractions*[a]

Cell fraction[b]	Incorporation of label (counts per min/mg dry wt of culture)	
	Bacillary form	L-form
Ethanol-soluble	300	1,850
Trypsin-solubilized	75	150
Residue	26,700	25

[a] The bacillary and L-forms were grown at 250 rev/min for 20 hr at 37 C in 10 ml of Penassay medium or T-medium, respectively, containing 0.1 μCi of α, ϵ-diaminopimelic acid-*1*,7-^{14}C per ml. The cultures were harvested and fractionated by the Park and Hancock procedure.

[b] Incorporation of label into the cold and hot trichloroacetic acid fractions is not shown.

TABLE 2. *Antibiotic sensitivities of the bacillary form and L-form*[a]

Antibiotic	Minimal growth inhibitory concentration (μg/ml)	
	Bacillus	L-form
Bacitracin	200	5
Chloramphenicol	2	2
D-Cycloserine	20	600
Novobiocin	1	0.2
Penicillin G	1	2,000+[b]
Vancomycin	0.5	600

[a] The bacillus and L-form were grown in 5 ml of Penassay medium or T-medium, respectively, containing serial dilutions of antibiotic. The lowest concentration of antibiotic able to inhibit growth was determined visually after incubation at 37 C for 48 hr at 60 rev/min.

[b] L-form growth was not inhibited at this concentration. Higher concentrations of penicillin G were not tested.

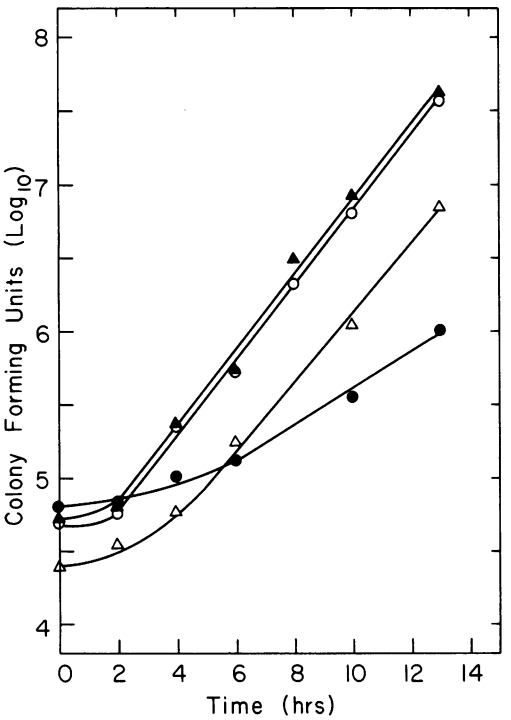

FIG. 2. *Growth of sal-1 in the presence of* D-*cycloserine. See legend of Fig. 1 for procedures. Growth was followed in T-medium with 1.2* M *NaCl: without antibiotic* (▲); *or with cycloserine at a concentration of 20 μg/ml* (○), *100 μg/ml* (△), *or 500 μg/ml* (●). *The curves are from duplicate experiments.*

which are specific inhibitors of peptidoglycan synthesis did not affect the growth of *sal-1* at the concentrations tested, which supports the analytical data.

Nucleotide-bound murein precursors were not detected in the cytoplasm of *sal-1*, even after cells from 1-liter logarithmic cultures were incubated at 37 C for 30 min in T-medium containing penicillin G (1,000 μg/ml), vancomycin (50 μg/ml), or bacitracin (5 μg/ml). Gross lysis of the L-form suspensions with loss of cytoplasmic components was not observed by phase microscopy during incubation in T-medium with or without antibiotics. The bacillary form incubated for 90 min in medium containing 20 μg of vancomycin per ml produced 26 nmoles of nucleotide-bound *N*-acetylhexosamine per mg dry weight of culture, which is similar to the amount found in *Staphylococcus aureus* strain H in this laboratory (6).

Structure of sal-1. The morphology of *sal-1* was studied by both phase and electron microscopy. As shown in Fig. 5, this organism grows as discrete spheres. Some of the spheres have a number of small bodies (SB) attached to their surface which are similar to those seen in bacillary cultures which are treated with lysozyme in the presence of osmotic stabilizers (11, 35, 42). In addition, the larger spheres frequently contain lighter areas suggestive of vacuoles. Introduction of distilled water under the cover slip results in rapid lysis, as noted previously with stabilized protoplasts of *S. aureus* (12).

Examination of thin sections in the electron microscope confirmed and extended the above observations. There were many examples of cells which appeared to be dividing by binary fission. In Fig. 6 the cytoplasmic membrane can be traced around most of the cell, indicating that this is not a tangental section. Cell wall remnants were not observed. Note that the nucleus in this section is polydispersed. A series of sections throughout the growth of a culture is shown in Fig. 7. The compact nuclear mass is

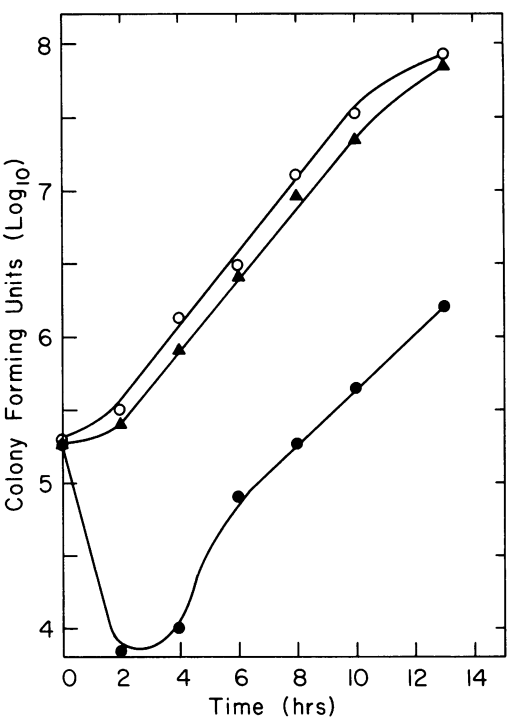

FIG. 3. *Effect of bacitracin on the growth of sal-1. Procedure is the same as outlined in Fig. 1, except that the overnight culture used for inoculum was diluted 100-fold. Viable count in T-medium alone* (○) *or with bacitracin at a concentration of 2 μg/ml* (▲) *or 4 μg/ml* (●). *Points are an average of duplicate experiments.*

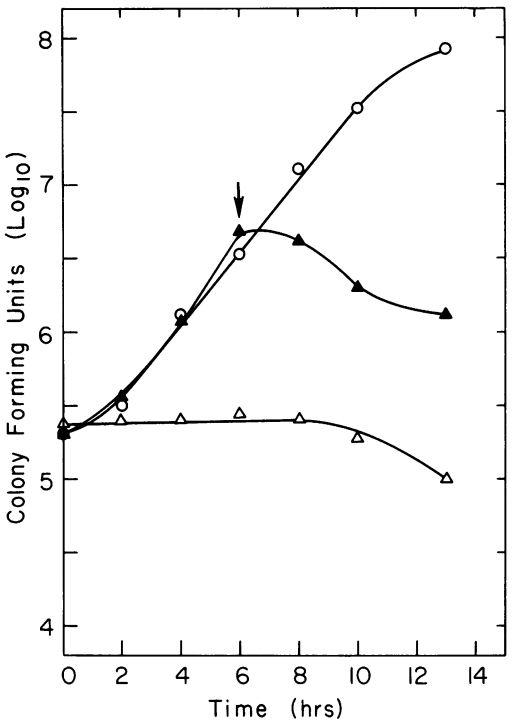

FIG. 4. *Inhibition of growth sal-1 by novobiocin. Procedure is the same as given in Fig. 3. Growth in T-medium alone* (○) *and in T-medium containing 0.2 μg of novobiocin per ml* (△). *At 6 hr novobiocin was added to a culture in T-medium to give a final concentration of 0.2 μg/ml* (▲).

more prominent in cells removed from logarithmically dividing cultures (Fig. 7a). The well-defined small bodies (SB) are surrounded by a cytoplasmic membrane. These are probably the small bodies which were observed in phase microscopy. In early stationary phase (Fig. 7b and c), the nucleus is usually polydispersed. Autolysis is frequently encountered (Fig. 7b). These autolyzing cells contain dense aggregates that resemble those described recently by Behme and Fitz-James (2). As shown in Fig. 7d, the cytoplasmic membrane has many electron-dense areas where small bodies are attached even in late stationary phase. Whether these merely represent segments of the cytoplasmic membrane that were pinched off remains to be established. As shown in Fig. 8, the vacuoles contain a number of smaller intracytoplasmic bodies. Many of these are probably interconnected and appear discontinuous only due to the plane of section. As shown in Fig. 9, a tangential section, some cells appear to give rise to buds; however, this is most probably an artifact of section. Thus, we have relied on the ability to trace the cytoplasmic membrane around the cell as an index of the plane of section.

The L-form colonies are composed primarily of single cells. Autolysis is a prominent feature in these colonies (Fig. 10). Occasionally the cells contain intracytoplasmic membranes. The significance of these structures is not known.

Comparison of bacillary and L-form membrane proteins. Since it was established that *sal-1* does not synthesize cell wall under the conditions described, it was of interest to compare the membrane proteins from protoplasts derived from lysozyme-treated bacilli with those from L-form membranes. SDS-solubilized membrane proteins were subjected to polyacrylamide disc gel electrophoresis in gels containing 7.5% acrylamide and 0.1% SDS, as described in Materials and Methods. A typical membrane protein profile obtained from SDS-treated protoplast membranes derived from a logarithmic culture of *B. subtilis* grown in Spizizen minimal medium is shown in Fig. 11a. Thirty bands are present. The relative amounts of protein in each band do not vary from preparation to preparation. This pattern corresponds closely to the protein profile obtained from protoplasts of *B. subtilis* by Taber et al. (38). Mid-logarithmic bacillary cultures in minimal medium supplemented with 1.2 M NaCl also were examined. The same number of bands were present, but their relative amounts were different (Fig. 11b). Apparently the concentration of NaCl in the growth medium affects the distribution of membrane proteins and should be considered when comparing proteins from organisms grown under different

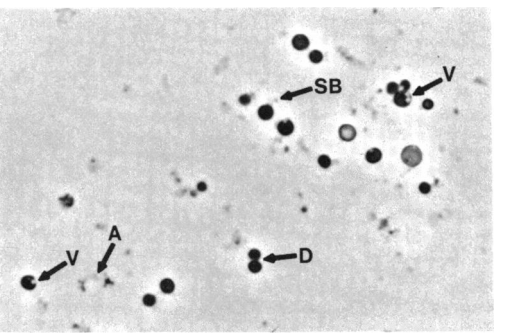

FIG. 5. *Morphology of sal-1. A representative field of sal-1 examined by phase microscopy demonstrates cells with small bodies (SB) and with vesicles (V). Another cell is shown that has autolyzed (A). The doublet (D) appeared to be attached, and the two spheres moved together with Brownian motion.*

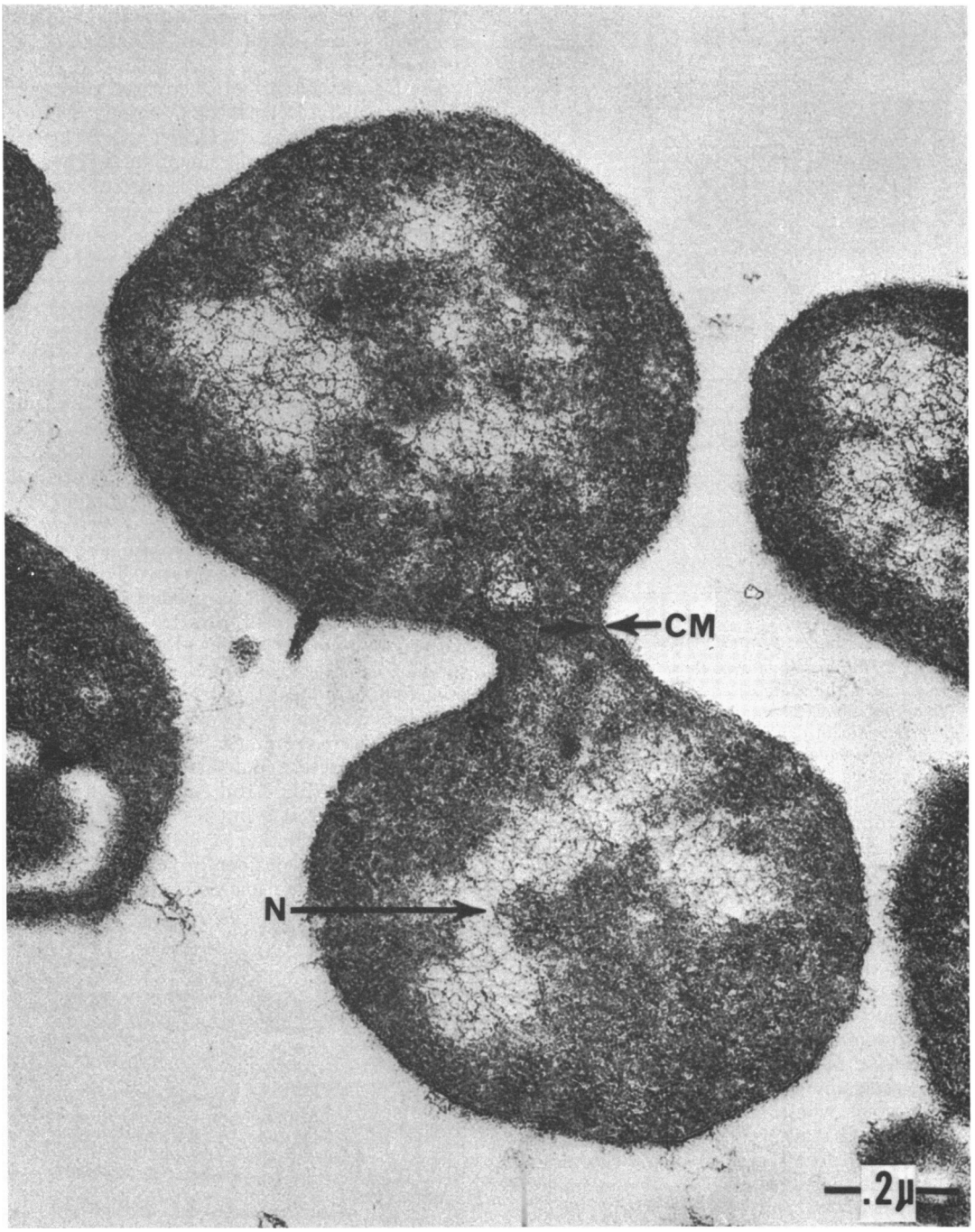

FIG. 6. *Morphology of sal-1. A representative doublet from a 16-hr culture of sal-1 in T-medium containing 1.2 M NaCl. Note the cytoplasmic membrane (CM) can be traced around approximately 80% of the circumference of the cell. The nucleus is denoted with N.*

conditions. Figures 11c and 11d represent gel patterns from L-form membrane proteins isolated from logarithmic cultures grown in T-medium containing 0.34 M or 1.2 M NaCl, respectively. Again, the concentration of NaCl in the medium caused quantitative changes in the distribution of protein. When the protein from the gel pattern of the bacillus is compared

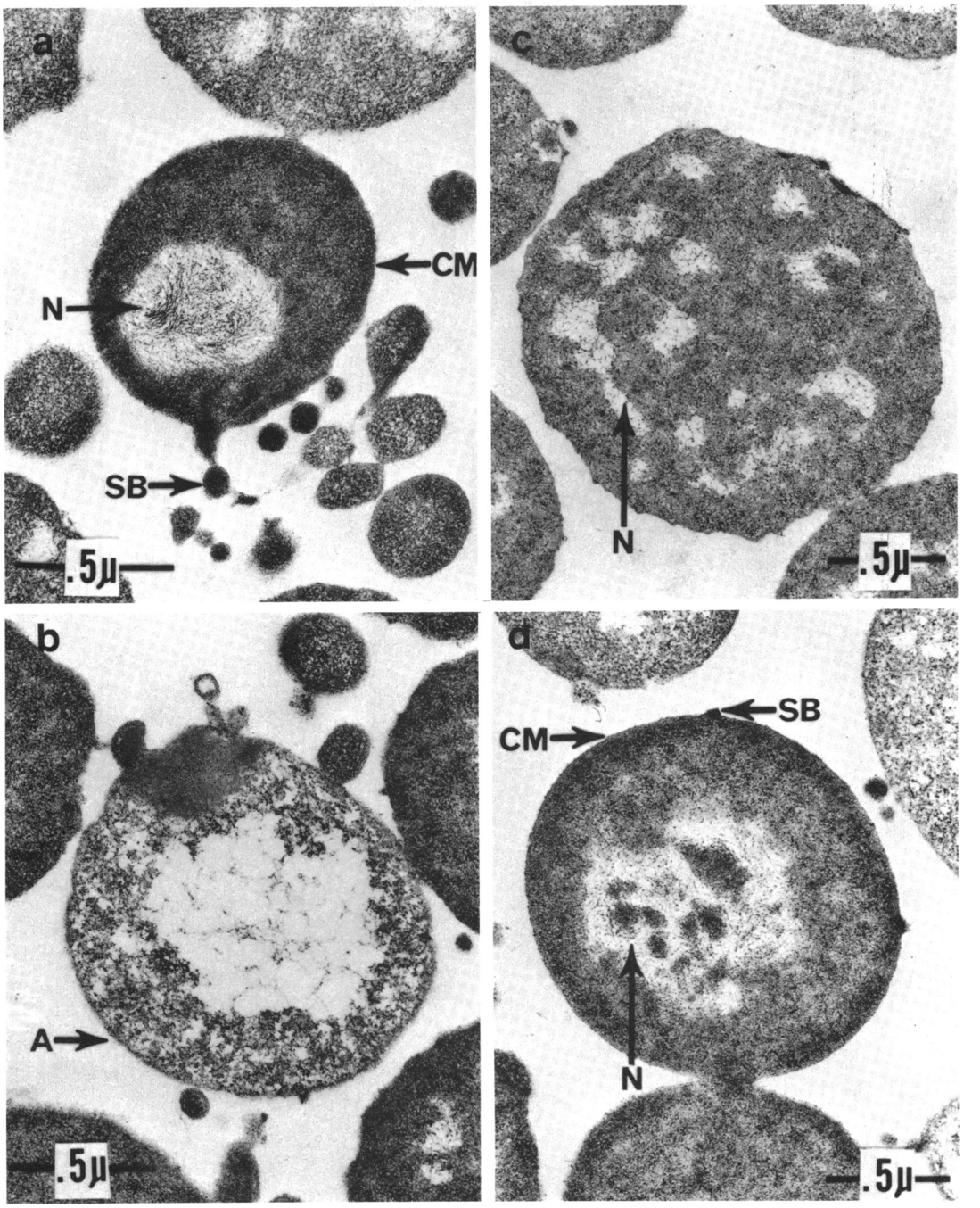

FIG. 7. *Morphology of sal-1 at various phases of growth. The cells were grown for various times in T-medium containing 1.2 M NaCl at 250 rev/min. a, 9.5 hr; b, 12 hr; c, 12 hr; and d, 14 hr. Symbols: A, autolysis; CM, cytoplasmic membrane; N, nucleus; and SB, small body.*

to the pattern from the L-form grown in medium containing 1.2 M NaCl (Fig. 11b and Fig. 11d, respectively), major differences in membrane components are apparent. Similar alterations are noted when the membrane proteins of the bacillus grown in normal medium (Fig. 11a) were compared to the proteins from the L-form grown in T-medium containing 0.34 M NaCl

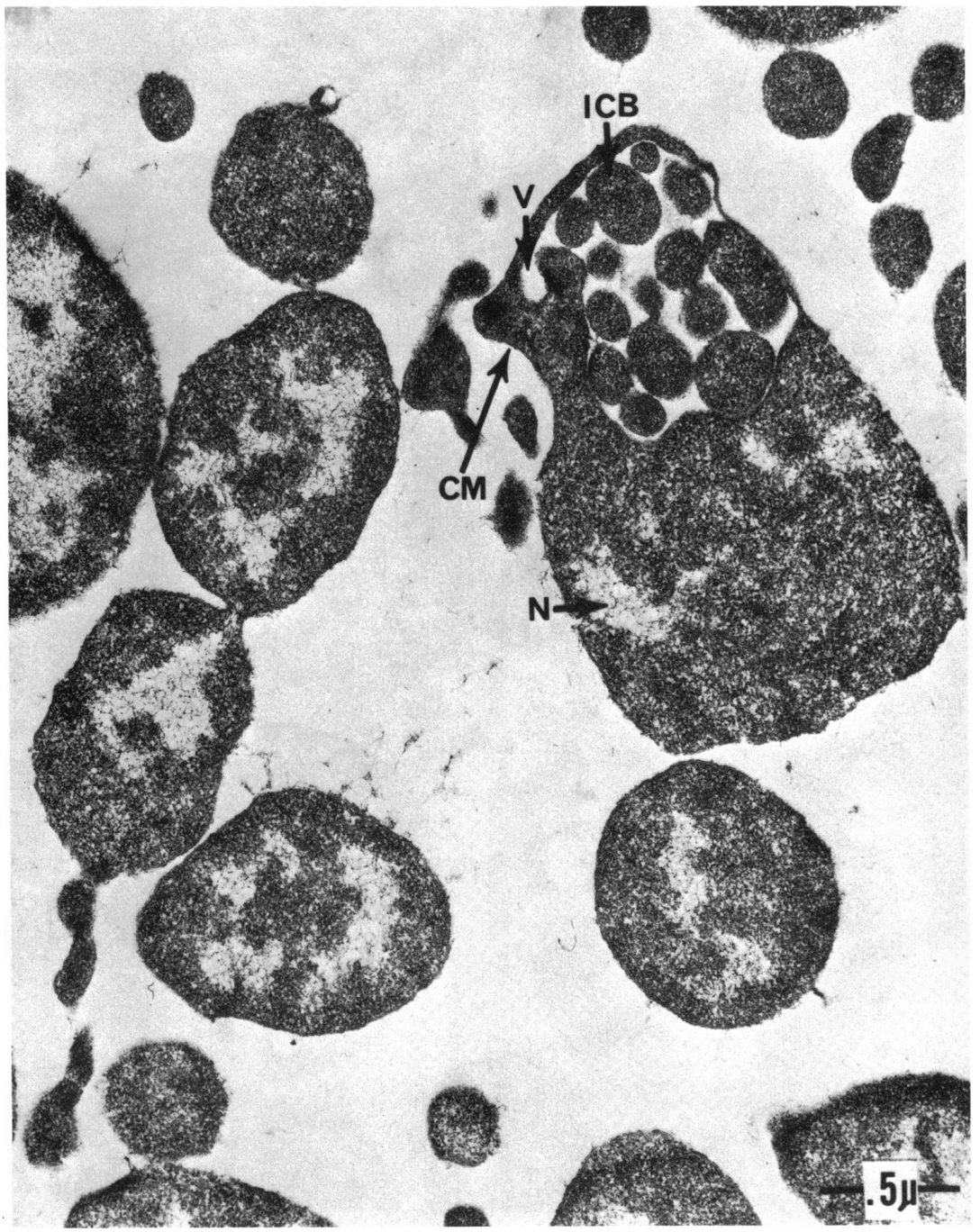

FIG. 8. *Morphology of sal-1 containing vesicles. A culture grown as described in Fig. 7 for 3 hr. Symbols: CM, cytoplasmic membrane; ICB, intracellular bodies; N, nucleus; and V, vesicle.*

(Fig. 11c). When the bacillary form was grown in T-medium instead of minimal salts medium, more dramatic differences in band patterns were found when compared to the L-form. Therefore, the conversion from bacillary to L-form morphology resulted in redistribution of membrane proteins.

DISCUSSION

This study has clearly demonstrated that

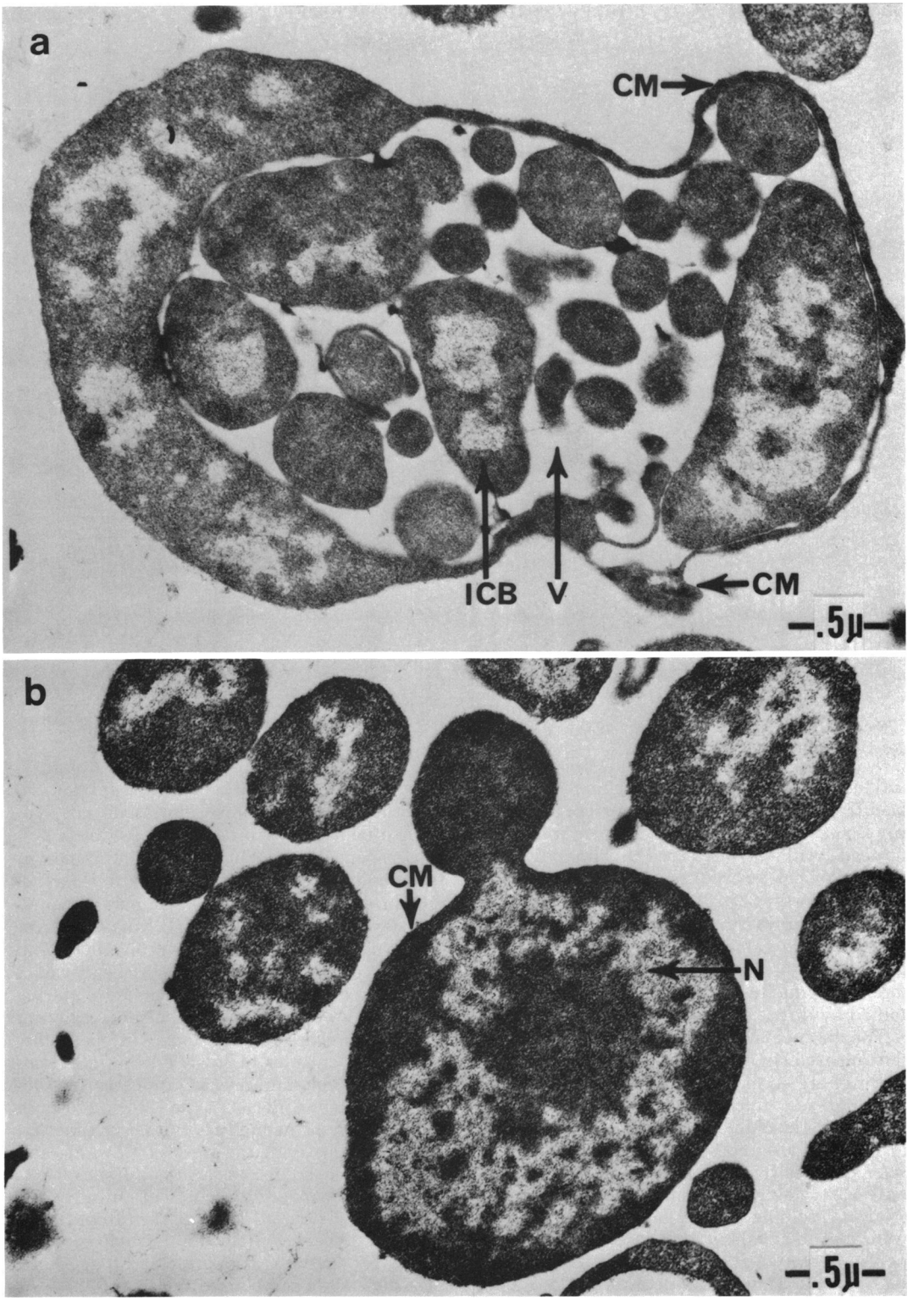

FIG. 9. *Morphology of sal-1 grown for 5 hr in T-medium. a, Large body containing intracellular bodies (ICB); b, tangential section through a cell suggestive of budding. Symbols as in Fig. 8.*

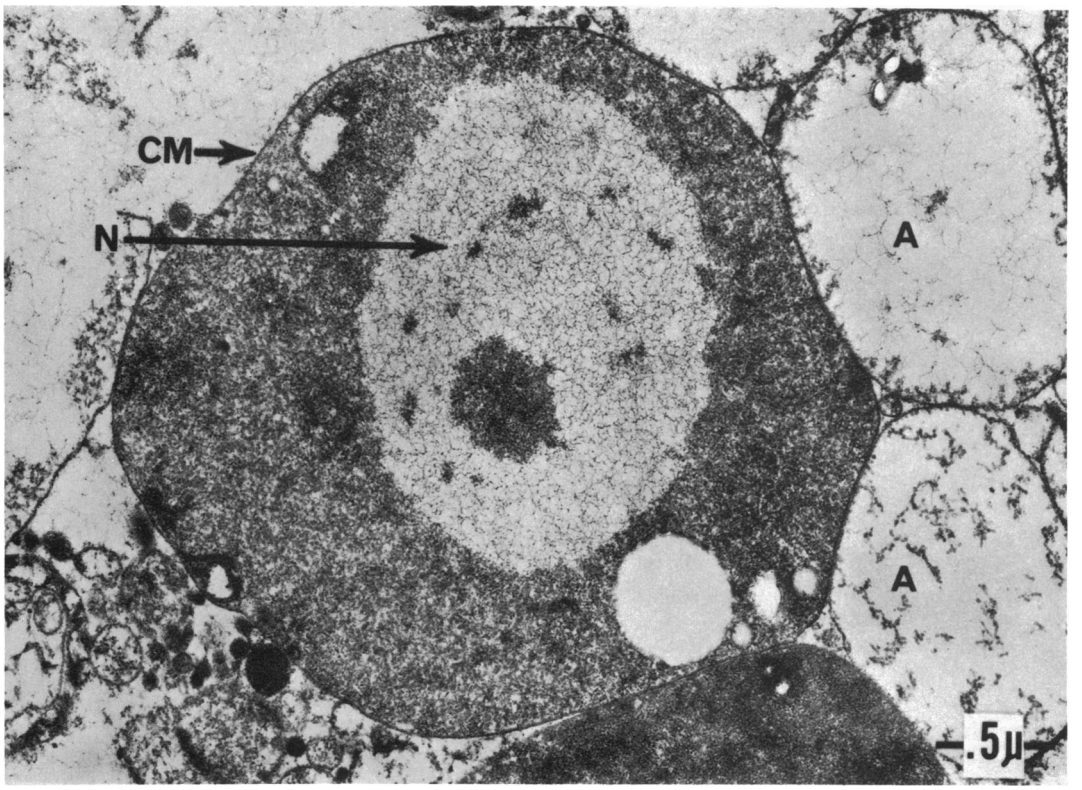

FIG. 10. *Morphology of L-form colony. A representative field of a colony grown for 4 days on agar as described in Materials and Methods.*

sal-1 is a stable L-form (7), since no reversion to bacillary morphology has occurred after 200 consecutive transfers in liquid medium. Also, no cell wall was detected by analytical or morphological techniques. This contrasts with earlier reports (22, 23) that soft agar must be present in growth medium for the multiplication of stable L-forms. No clumping is visible during growth of *sal-1* in liquid medium. Therefore, reproducible growth curves can be obtained by direct counts or viable count.

The absence of cell wall in stable L-forms is documented in the literature (31, 35). Our findings are similar to those of King et al. (18) who did not find cell wall hexosamines in formamide-insoluble extracts from a stable L-form of *Streptococcus faecium*. Fractionation of *sal-1* cultures by the Park and Hancock procedure (32) yielded several amino acids in the trypsin-insoluble residue, but their molar ratios were not equivalent to those found in the peptidoglycan of *B. subtilis* (46). Dpm, which is found exclusively in the peptide chain of peptidoglycan (44), was not present in the trypsin-insoluble residue of L-form cells. Ryter and Landman (35) and Clive and Landman (7) hypothesized that stable L-forms do not synthesize peptidoglycan because a feedback system responsible for continuous cell wall biosynthesis is interrupted, possibly due to a lack of membrane acceptor sites or membrane-bound transfer enzymes, or both (31). If nascent peptidoglycan were synthesized but unable to bind to an acceptor site on the cell surface, it would be released into the culture medium during growth. However, neither peptidoglycan nor cell wall precursors were found in spent medium from cultures of *sal-1*. Furthermore, nucleotide-bound muramyl peptide was not detected in the cytoplasm of the L-form grown in the presence of penicillin or other antibiotics. This contrasts with the work of Edwards and Panos (10) who found uridine diphosphate-muramic acid-peptide accumulation in the cytoplasm of a stable L-form derived from *Streptococcus pyogenes* AED (36). Obviously, the reason for the lack of peptidoglycan synthesis may not be the same for all L-forms.

It is important to note that the phenotype observed in *sal-1* could result from a block in

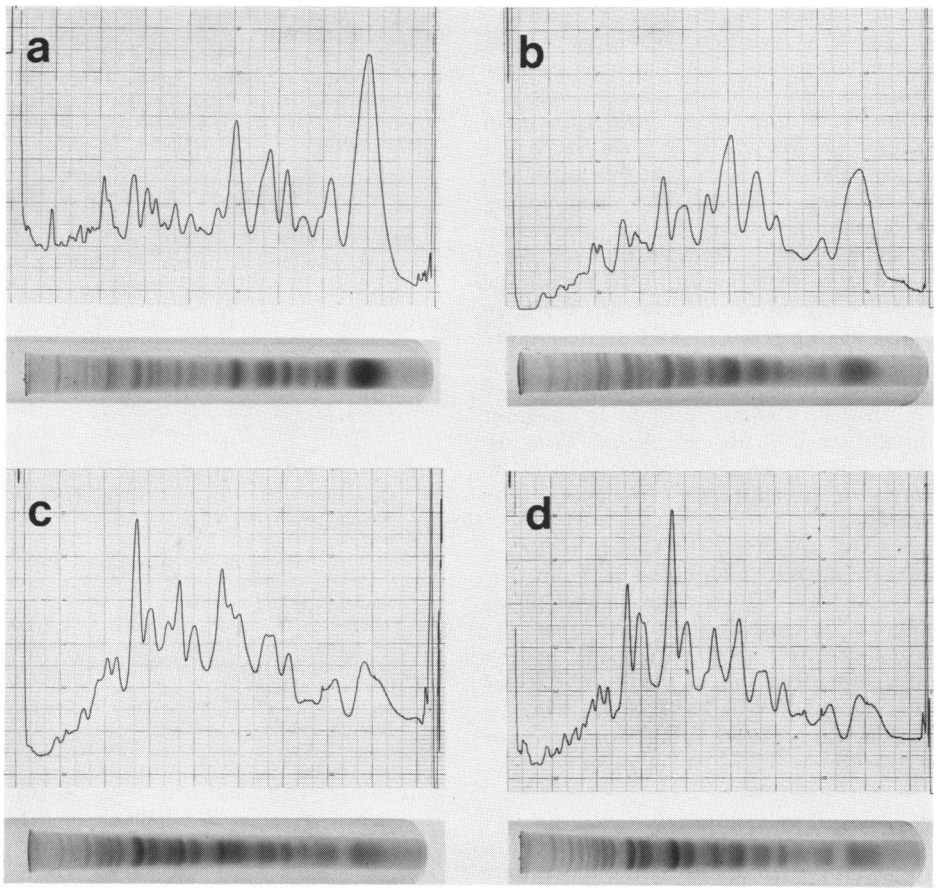

FIG. 11. *Membrane profile of bacillary and L-form. Gel electrophoresis patterns of protoplast membranes from bacillary cultures grown in: a, minimal medium, and b, minimal medium containing 1.2 M NaCl. Gel electrophoresis patterns of L-form membranes from L-forms grown in: c, T-medium containing 0.34 M NaCl, and d, T-medium containing 1.2 M NaCl. The procedures for electrophoresis, staining, and gel scan used are described in Materials and Methods.*

any of the enzymes involved in the biosynthesis of peptidoglycan or a defect in an essential pathway required for reversion from the L to the bacillary phase. Furthermore, this strain has been adapted for growth in liquid medium with variable concentrations of salt. The exact nature of the biochemical defect and its relation to the observation that removal of the cell wall decreases the biosynthesis of Dpm containing polymers (C. Bond and O. E. Landman, Bacteriol. Proc., p. 35, 1970) remain to be elucidated.

L-forms are frequently produced experimentally by treatment of the bacillary forms with antibiotics that inhibit cell wall biosynthesis (8, 36) and by enzymes that degrade the cell wall (22, 46). Consequently, the synthesis of cell walls by L-forms has been probed with antibiotics (17, 20). We found that antibiotics that are specific inhibitors of cell wall biosynthesis, such as penicillin (16, 40) or D-cycloserine (28), do not inhibit the growth of *sal-1* at physiological concentrations. The L-form was not sensitive to vancomycin. This was not unexpected since vancomycin inhibits in vitro synthesis of peptidoglycan by membranes from a stable L-form of *S. aureus* (5) and by protoplast membranes of other bacteria presumably in one of the steps of polymerization (25, 29). A lower concentration of novobiocin was needed to prevent growth of the L-form than the bacillary form. Novobiocin has been characterized as an inhibitor of cell wall synthesis (27), but Brock and Brock (3) reported that novobiocin affected the membrane permeability of L-forms of *Escherichia coli* K-12 that were induced by penicillin. When a growth-inhibitory concentration of novobiocin

was added to a logarithmically growing culture of *sal-1*, growth stopped, but viability did not drop immediately (Fig. 4). This suggests that membrane permeability was affected, but not enough to cause immediate lysis. Bacitracin inhibited *sal-1* at low concentrations. Stone and Strominger (37) have shown that this antibiotic inhibits cell wall biosynthesis by complexing with a membrane-bound C_{55}-polyisoprenoid pyrophosphate. They suggested that the complex may distort the structure of the membrane so that other functions are affected. We have not looked for this lipid in *sal-1* to determine whether the antibiotic binds to this lipid. It is interesting that the viability of cultures of *sal-1* recovers after incubation for 4 hr in medium containing 4 μg of bacitracin per ml. Whether recovery is due to selection of antibiotic-resistant clones or to adaptation remains to be investigated.

Electron microscopy of the L-form reveals a discrete cytoplasmic membrane with no evidence of cell wall. The combination of phase and electron microscopy indicates that the L-form divides by binary fission and not by disorganized constriction, as reported by Ryter and Landman (35). Whether the small bodies on the surface are related to mesosomes (11) or merely aggregated fragments of membrane remains to be elucidated.

Chemical analysis and the antibiotic sensitivities of *sal-1* suggested alterations in protein of the cytoplasmic membrane. We found differences between the membrane proteins of the L-form and bacillary form by SDS-polyacrylamide gel electrophoresis which could not be traced to changes in composition of the growth medium. Theodore et al. (39) identified the genera of L-forms by comparing their electrophoretic patterns to phenol extracts prepared from whole parental cells. The dissimilarity of band patterns between the bacillus and L-form in our study made identification of the parentage of *sal-1* difficult. Since Inouye and Guthrie (15) have developed procedures to detect a change in one membrane component of a temperature-sensitive mutant of *E. coli* which was defective in synthesis of deoxyribonucleic acid, it is hoped that our procedure could be refined to demonstrate whether these are differences in the cytoplasmic membrane which are related to biosynthesis of the cell wall. In addition, studies are in progress to determine whether the L-form membrane contains the enzymes that are associated with the biosynthesis of the cell wall.

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